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Deletion of *Candida albicans SPT6* Is Not Lethal but Results in Defective Hyphal Growth

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Abstract

As a means to study surface proteins involved in the yeast to hypha transition, human monoclonal antibody fragments (single chain variable fragments, scFv) have been generated that bind to antigens expressed on the surface of *Candida albicans* yeast and/or hyphae. A cDNA expression library was constructed from hyphae, and screened for immunoreactivity with scFv5 as a means to identify its cognate antigen. A reactive clone contained the 3′ end of the *C. albicans* gene, *orf 19.7136*, designated *SPT6* based on homology to *S. cerevisiae*, where its product functions as a transcription elongation factor. A mutant containing a homozygous deletion of *SPT6* was isolated, demonstrating that unlike *S. cerevisiae*, deletion of this gene in *C. albicans* is not lethal. Growth of this strain was severely impaired, however, as was its capacity to undergo filamentous growth. Reactivity with scFv5 was not detected in the mutant strain, although its impaired growth complicates the interpretation of this finding. To assess *C. albicans SPT6* function, expression of the *C. albicans* gene was induced in a defined *S. cerevisiae spt6* mutant. Partial complementation was seen, confirming that the *C. albicans* and *S. cerevisiae* genes are functionally related in these species.

Index descriptors

Candida albicans; *Saccharomyces cerevisiae*; single-chain variable fragment; hyphae

Introduction

Candida albicans is a well recognized human pathogen that causes both mucocutaneous and systemic infections primarily in immunocompromised hosts (Calderone, 2002). Systemic infections caused by this organism have increased in frequency and carry a high mortality despite antifungal therapy (Benjamin, et al., 2006; Viudes, et al., 2002). The capacity of this organism to shift its morphology from yeast to hyphal form is important for its virulence and has been the subject of intensive study (Calderone, 2002; San-Blas, et al., 2000). The shift to hyphal growth is marked by significant changes in gene expression and expression of novel surface antigens, and some of these have been implicated in interaction with the host and virulence (Kumamoto and Vinces, 2005).

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Because of its importance in disease states, several approaches have been used to probe specifics of the yeast to hypha transition. Traditional genetic approaches have been hampered by the diploid nature of *C. albicans*, and unlike the related yeast, *Saccharomyces cerevisiae*, the lack of a well defined sexual cycle. *S. cerevisiae* can be induced to grow in a pseudohyphal form, and *C. albicans* homologues to the genes involved in pseudohyphal growth have been studied (Leberer, et al., 1996; Liu, et al., 1994). Screening *C. albicans* gene libraries for their capacity to elicit pseudohyphal growth in *S. cerevisiae* has also met with some success (Feng, et al., 1999; Kadosh and Johnson, 2001; Stoldt, et al., 1997). Another fruitful approach involved large-scale transposon mutagenesis of *C. albicans* with selection of clones that had altered hyphal phenotypes (Uhl, et al., 2003). The yeast to hypha transition is also amenable to study via genomic microarray. Such an approach has identified 61 genes induced and 25 genes repressed in response to exposure to serum at 37°C (Kadosh and Johnson, 2005).

As the outermost structure, the cell wall is in closest contact with host defense mechanisms during infection and modulates the host-pathogen interaction. As such, defining immunogenic cell wall components and the capacity of antibody specific to these components to be protective has received much study. Screening of sera from both human and animals infected with *C. albicans* for specific antibodies has defined gene products from the cell wall as well as cytoplasmic and secreted proteins that elicit an antibody response. Antibodies against some of these proteins are well documented to have protective properties (Lopez-Ribot, et al., 2004). More recently, sophisticated proteomic and bioinformatic approaches have also been applied to identify gene products of the organism that elicit potentially protective antibody responses from the host. Studies comparing substantive collections of sera from patients with systemic candidiasis compared to controls have demonstrated unique signatures between the commensal and disease state that have both diagnostic and therapeutic implications (Pitarch, et al., 2006). Components of an effective cell wall extract vaccine that were associated with protective responses have also been identified using a proteomic approach (Thomas, et al., 2006). Advances in technology have also allowed systematic genomic analyses to be applied to determine gene products of the organism that are preferentially expressed under *in vivo* conditions. Potential virulence factors have been identified by methods including differential display, signature-tagged mutagenesis, transcriptional profiling by microarray, and antibody based screening strategies (Nguyen, et al., 2004). This approach has identified novel virulence factors and allows additional insights into the organism's pathogenesis and the impact of varied host environments (Cheng, et al., 2005).

As a means to obtain additional reagents to explore the antigenic milieu of the hyphal surface and potentially identify novel proteins that may have a role in the organism's virulence, we used phage display technology to isolate human antibody fragments (single-chain variable fragments, scFv) that are reactive with both the yeast and/or hyphal form of *C. albicans* (Bliss, et al., 2003; Haidaris, et al., 2001). To identify clones specific for *C. albicans* surface antigens expressed under native conditions, the human scFv phage display library was panned against live, whole cells growing in either the yeast or germ tube morphology. These scFv have been shown to facilitate interaction between the fungus and host immune cells (Wellington, et al., 2003). Additionally, one of these scFv (scFv3) recognizes the well-characterized fungal adhesin, Als3p, on the hyphal surface and interferes with Als3p mediated adhesion to human epithelial and endothelial cells *in vitro* (Laforce-Nesbitt, et al., 2008). The cognate antigen of another hyphal-specific scFv (scFv5) has remained elusive. To identify candidate gene products that may be the target of scFv5, a cDNA expression library from *C. albicans* hyphae was constructed and screened. We report the identification of the as yet poorly characterized *C. albicans* gene product, Spt6p, as a potential target for scFv5 and the characterization of a strain harboring a homozygous *SPT6* deletion.

Materials and Methods

Strains and media

Strains used in this study are listed in Table 1. Media used include YPD (1% yeast extract, 2% peptone, 2% dextrose), Agar + serum (1% agar, 4% fetal calf serum), and spider medium (1% nutrient broth, 1% mannitol, 0.4% potassium phosphate, 1.3% agar, pH 7.2). Yeast dropout media for strains with auxotrophic markers were prepared by standard methods (Sambrook and Russell, 2001).

Construction and immunoscreening of *C. albicans* hyphae cDNA expression library

Starter cultures of *C. albicans* strain SC5314 were grown 16 h at 37°C with vigorous agitation in YPD medium. Cultures were predominantly (>99%) yeast forms following this incubation. Cells were washed and resuspended at 5×10^5 cells/ml in Medium 199 and incubated at 37°C for 24 h to induce hyphal growth. A cDNA expression library in λTriplEx2 (Clontech, Mountain View, CA) was constructed from hyphae by Bio S&T (Montreal, Quebec). Approximately 1.5×10^6 primary recombinant clones were amplified in 15 pools. Each pool was grown as plaques in an *E. coli* (XL-1 Blue) top agar lawn on 10 – 150 mm petri dishes, with approximately 1000 plaques per dish according to the manufacturer's instructions. Expression of cDNA insert was achieved by induction with isopropyl-β-D-thiogalactopyranoside (IPTG). Plaques were transferred to nitrocellulose and filters were blocked with 3% skim milk. Filters were probed with scFv5 prepared as described previously (Laforce-Nesbitt, et al., 2008), washed, and scFv5 binding was detected with mouse anti-FLAG antibody (Sigma) followed by alkaline phosphatase conjugated secondary antibody and chromogenic substrate. Reactive plaques were picked from the original top agar overlay and amplified. Successive rounds of immunostaining were done to confirm continued reactivity with scFv5 and to assure clonality. A positive phage clone (designated clone 3-7) was converted to plasmid (designated pBMJ9) following manufacturer's instructions and the insert sequenced using manufacturer's sequencing primers.

Construction of homozygous *SPT6* deletion mutant in *C. albicans*

A homozygous deletion in *SPT6* was derived from *C. albicans* strain SN87 (Noble and Johnson, 2005). Sequences for oligonucleotides used in this study are listed in Table 2. PCR products for targeting the *SPT6* open reading frame (ORF) were generated using oligonucleotides 1 and 2 to amplify the 5′ flank of *SPT6* and oligonucleotides 3 and 4 to amplify the 3′ flank *of SPT6*. Selectable marker sequences (*Candida dubliniensis HIS1* and *Candida maltosa LEU2*) were also amplified from plasmids pSN52 and pSN40, respectively, as previously described (Noble and Johnson, 2005). Fusion PCR products were generated by using oligonucleotides 1 and 4. These PCR primers amplify the flanking sequences of *SPT6* together with either the *HIS1* or *LEU2* marker PCR product (Noble and Johnson, 2005). The first allele of *SPT6* was replaced using the *HIS1* marker in strain SN87 (designated JMB220). The second allele of *SPT6* was replaced with the *LEU2* marker to generate a homozygous knockout of this gene (designated JMB31). Correct integration of the PCR products was verified by PCR across the 5′ disruption junction using oligonucleotides 5 and 6 (*HIS1* marker) or 5 and 7 (*LEU2* marker), and across the 3′ disruption junction using oligonucleotides 8 and 9 (*HIS1* marker) or 8 and 10 (*LEU2* marker). Loss of the ORF was confirmed using PCR primers internal to the *SPT6* ORF (oligonucleotides 11 and 12).

Southern analysis was also performed to confirm loss of *SPT6* in JMB31. Genomic DNA was prepared from SN87 (parent), JMB220 (heterozygote) and JMB31 (Δ*spt6*/Δ*spt6*) using a yeast DNA purification kit (Epicentre Biotechnologies) according to maufacturer's instructions and digested with ScaI. DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membrane by standard methods (Sambrook and Russell, 2001). A 197

nucleotide probe was generated by PCR using oligonucleotides 13 and 2, gel purified, and labeled with ^{32}P-dCTP using the random primers method. The blot was hybridized overnight at 65°C, washed, and bound probe was detected by digital phosphorimaging.

To construct a strain containing a reintegrated copy of *SPT6*, the gene was amplified by PCR using oligonucleotides 14 and 15, which incorporate an *Apa*I and *XhoI* site, respectively. The full length gene was then cloned into the corresponding sites of pSFS2a (Reuβ, et al., 2004). This construct contained the dominant nourseothricin resistance marker, *SAT1*, which allowed positive selection of transformants. The construct was linearized with *Eco*NI and transformations were conducted with the knockout strain, JMB31, and plated on YPD + nourseothricin. No transformants were obtained despite multiple attempts, suggesting that the impaired growth of JMB31 may adversely affect its capacity for transformation. To circumvent this issue, a strategy was devised to allow replacement of the native *SPT6* with a truncated form in the heterozygote, JMB220, while simultaneously placing the gene under control of the inducible *MET3* promoter (Fig. 1). First, to allow positive selection with nourseothricin, the *SAT1* gene was excised from pSFS2a using *Eco*RI and *Pst*I and cloned into the corresponding sites of pCaEXP (Care, et al., 1999), designated pBMJ40. Next, a fragment containing the 5′ end of *SPT6* (615 bp) was amplified by PCR in two steps to incorporate a unique *Sac*I restriction site in the middle of the fragment. Creation of the *Sac*I site required 2 nucleotide changes relative to the native *SPT6* ORF, but both were translationally silent. Oligonucleotides 16 and 17, or 18 and 19, were used to amplify the 5′ and 3′ ends of the 615 bp fragment, respectively. Oligonucleotides 17 and 18 contain a *Sac*I site and are complementary, allowing these fragments to be combined in a second PCR with primers 16 and 19 to generate the 615 bp fragment with flanking *BamH*I and *Pst*I sites. This fragment was cloned into the corresponding sites of pBMJ40, and designated pBMJ41. This plasmid was then linearized with *Sac*I, transformed into the *SPT6* heterozygote, JMB220, and plated on YPD + nourseothricin. Correct integration of the construct was verified by PCR across the 5′ integration junction using oligonucleotides 13 and 20 and across the 3′ disruption junction using oligonucleotides 21 and 22. These PCR products confirmed that the native *SPT6* promoter was adjacent to the truncated *SPT6* gene and the *MET3* promoter was adjacent to the full length *SPT6* gene. The reintegrant strain was designated JMB222.

Indirect immunofluorescence assay (IFA) of *C. albicans* strains with scFv5

IFAs of SN87 (parent), JMB31 (Δ*spt6*/Δ*spt6*), and JMB222 (Δ*spt6*/*spt6**-*SPT6*) were conducted on glass coverslips using scFv as described previously (Bliss, et al., 2003).

Complementation of *S. cerevisiae SPT6* mutant

C. albicans SPT6 was amplified by PCR from wild-type strain SC5314 using oligonucleotides 23 and 24, which incorporate a *Spe*I and *Xho*I site, respectively. The 4.2 kb fragment was cloned in the corresponding sites of the *S. cerevisiae* expression vector, p416-GAL1 (Mumberg, et al., 1994) to create plasmid pBMJ10. This plasmid was transformed into *S. cerevisiae* strain FY137 (Hartzog, et al., 1998), which contains a mutation in *SPT6* that leads to temperature sensitivity and suppression of auxotrophic markers for histidine and leucine. The parent vector, p416-GAL1 was transformed as a negative control, and a positive control plasmid containing *S. cerevisiae SPT6* under control of the *GAL1* promoter, pBY011 (generously provided by Fred Winston) was also included. Growth phenotypes for FY137 containing each of the 3 plasmids were compared at 30°C on drop-out solid media lacking uracil (selectable marker for the plasmid) and either leucine or histidine, and on drop-out solid media lacking uracil at 37°C to test temperature sensitivity.

Results

Screening of a *C. albicans* hyphae expression library for immunoreactivity with scFv5

Messenger RNA from *C. albicans* hyphae was used to construct a cDNA expression library in bacteriophage λ. The library contained 1.5×10^6 independent clones which carried an average insert size of >1.2 kb. The library was grown as plaques on an *E. coli* lawn under conditions leading to expression of insert, and plaque lifts were performed. Filters were then probed for reactivity with scFv5, and positive plaques further purified. A reactive clone was purified to homogeneity and the insert sequenced. This clone (designated 3-7) contained 1951 bp homologous to the 3′ end of the gene identified as *orf 19.7136* (*SPT6*) in the *Candida* genome database (Arnaud, et al., 2007). The 1951 bp insert represents 46% of this 4206 bp gene. Comparison of clone 3-7 sequence with the database sequence demonstrated 99% sequence identity. There were a total of 9 nucleotide and 6 amino acid discrepancies with the published sequence. DNA sequence data for clone 3-7 was deposited with GenBank (accession number **GQ337860**).

The published sequences of *C. albicans SPT6* and *S. cerevisiae SPT6* were compared by Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). At the protein level, these sequences are 34% identical and 55% similar. Additionally, *C. albicans* Spt6p was found to contain the Src Homology 2 (SH2) domain that has been noted in Spt6 proteins of other organisms (Fig. 2) and is the only known SH2 domain in the yeast genome (Maclennan and Shaw, 1993).

Deletion of *C. albicans SPT6*

To establish whether the cognate antigen for scFv5 was the *SPT6* gene product, a homozygous deletion mutant was constructed, designated JMB31. PCR with primers spanning both the 5′ and 3′ disruption junctions confirmed integration of the disruption constructs at the predicted locus and loss of the *SPT6* open reading frame as described in Materials and Methods (data not shown). Deletion of *SPT6* in this strain was also confirmed by Southern analysis (Fig. 3). Unlike *S. cerevisiae*, deletion of this gene in *C. albicans* was not lethal. However, the mutant's growth was markedly abnormal, and it was temperature sensitive. No growth occurred at 37° C, and growth at 30°C was very slow (Fig. 4). Additionally, the mutant had a severe defect in hyphal growth (Fig. 5). No hyphae were detected on solid media containing serum or on spider media. Microscopically, after a 6 hour incubation in Medium 199, the defective hyphal growth was readily apparent. To test reactivity with scFv5, immunofluorescence assays were conducted on JMB31 and its wild-type parent. No reactivity with scFv5 was detected on the mutant strain (Fig. 6). However, because the morphology of the mutant was drastically altered, the lack of binding may or may not reflect deletion of the target antigen. Disordered growth of the structure on which the target antigen is normally displayed is a reasonable alternative conclusion.

To establish a complete analysis of the mutant phenotype, reintegration of the wild-type *SPT6* gene was attempted in the knockout strain JMB31. The gene was cloned into pSFS2A (Reuβ, et al., 2004) and DNA sequencing was used to confirm that the construct was appropriate. However, despite multiple attempts to transform the construct into JMB31, no transformants containing the reintegrated gene were obtained, while control transformations proceeded with expected efficiency. The significantly impaired growth phenotype of JMB31 likely compromises its capacity to undergo transformation. To circumvent this issue, a strategy was devised (Fig. 1) to replace the 2nd copy of *SPT6* in the heterozygote with a truncated gene and simultaneously place the full length *SPT6* gene under control of *the MET3* promoter (Care, et al., 1999). PCR using primers that spanned the predicted integration site confirmed that the native *SPT6* had been replaced with the truncated version and that full length *SPT6* was adjacent

to the *MET3* promoter. Although transcription from *the MET3* promoter is repressed in the presence of methionine or cysteine in the culture media (Care, et al., 1999), the *spt6* mutant phenotype could not be recapitulated in the reintegrant strain regardless of growth conditions. Presumably, *the MET3* promoter or other factors within the locus allow enough transcription of *SPT6* to correct for the severe growth defect in the *spt6* deletion mutant. Nevertheless, because truncation of native *SPT6* was confirmed, the phenotype of the reintegrant strain was analyzed. This strain had a growth rate comparable to wild-type (data not shown) and had no defect in capacity for hyphal growth (Fig. 5). It is likewise reactive with scFv 5 by IFA (Fig. 6).

Complementation of a *S. cerevisiae SPT6* mutant

The *S. cerevisiae SPT6* gene product functions as a transcription elongation factor, acting to modulate chromatin structure and prevent transcription from promoters within coding regions (Bortvin and Winston, 1996; Kaplan, et al., 2003). Deletion of *SPT6* is lethal in *S. cerevisiae* (Clark-Adams and Winston, 1987), although mutants have been described that suppress defects in *HIS4* and *LEU2* (Winston, et al., 1984). FY137 contains such a mutation, *spt6-140*, that results in temperature sensitivity (ts) and suppresses *his4* and *leu2* mutations of the parent strain. Thus, its phenotype is ts, his+, leu+. Because *spt6-140* is recessive, expression of wild-type *S. cerevisiae SPT6* in trans restores the temperature resistant (tr), his-, leu- phenotype of the parent (Fig. 7). To test whether *C. albicans SPT6* is capable of suppressing the *S. cerevisiae* mutant, the full length gene was cloned into a *S. cerevisiae* expression plasmid under the control of the *GAL1* promoter. Proper in frame insertion and orientation of the insert was confirmed by DNA sequencing. Growth phenotypes on histidine or leucine drop-out media containing galactose to induce *SPT6* expression, and growth at 37°C were determined (Fig. 7). Expression of the *C. albicans* gene partially suppressed the mutant phenotype, based on inhibition of growth on histidine drop-out media and restoration of growth at 37°C. However, the growth phenotypes were intermediate between the conditional mutant strain and complementation by *S. cerevisiae SPT6*.

Discussion

The yeast to hypha transition in *C. albicans* is a complex, carefully orchestrated process in which the physiological state of the organism is significantly altered by the programmed activation of a subset of genes. We have examined scFv specific for the hyphal form of the organism as a means to identify potentially novel antigens expressed on the cell surface as part of this transition. Our screen of a hyphal cDNA expression library for clones reactive with hypha-specific human antibody fragments has identified the *SPT6* gene product as the possible cognate antigen for scFv5. The altered physiology of a strain deleted for this gene has made definitive conclusions about its product's reactivity with scFv5 difficult to obtain. ScFv5 does not bind to the mutant, but this observation may reflect the defective hyphal growth *in* th*e* strain rather than the specific lack of the cognate antigen.

We confirmed deletion of *SPT6* in the mutant by PCR as well as Southern blot. Although Western analysis would also be helpful to confirm deletion at the protein level, this experiment was not possible due to technical limitations. Previous attempts have been made to extract the antigen reactive with scFv5 from wild-type *C. albicans* using multiple biochemical methods. These studies have never yielded antigen reactive with scFv5 in a soluble form that is amenable to Western blotting, either under denaturing or native conditions. We concluded from these studies that the protein either exhibits poor solubility, or that scFv5 recognizes a conformational epitope that is difficult to maintain following extraction.

The *C. albicans gene, orf 19.7136*, has been designated *SPT6* based on homology to the *S. cerevisiae* gene (www.candidagenome.org). In *S. cerevisiae, SPT6* codes for a transcription

elongation factor involved in direct interaction with histones and mediation of chromatin structure (Bortvin and Winston, 1996). Although overall homology between the *C. albicans* and *S. cerevisiae* Spt6 proteins is somewhat limited (34% identical and 55% similar), they do bear some additional similarities. The predicted proteins are of similar size; *C. albicans* 1402 amino acids, *S. cerevisiae* 1452 amino acids. Both proteins have an acidic amino terminus, characteristic of proteins that interact with chromatin. In *S. cerevisiae*, the first 70 residues have a net charge of -30 and the amino terminal third of the protein has a net charge of -81 (Swanson, et al., 1990), whereas in *C. albicans* the net charge of these regions is -15 and -57, respectively. Additionally, the *C. albicans* Spt6p possesses a SH2 domain. This domain is important for the protein's interaction with RNA polymerase II (Pol II), as it associates with phosphoserine on the C-terminal repeat domain of the largest Pol II subunit (Andrulis, et al., 2000; Kaplan, et al., 2000). This observation provides strong evidence that the *C. albicans* gene is truly homologous to the gene in other organisms, as the SH2 domain in Spt6p is the only known SH2 domain encoded in the yeast genome. We also found evidence of similarity at a functional level. Inducing expression of the *C. albicans* gene in a well defined mutant of *S. cerevisiae* resulted in partial complementation of the mutant phenotype. Although the phenotype was less robust than that achieved with expression of *S. cerevisiae SPT6*, such a result is not surprising given the loose homology between the two organisms. Based on these observations, the most likely interpretation is that the *C. albicans* gene has a function homologous to that of *S. cerevisiae*. To our knowledge, however, this is the first report of an organism in which deletion of *SPT6* is not lethal.

Direct experimental evidence for the function of Spt6p in *C. albicans* is generally unavailable. Several studies using genomic screening approaches to study an aspect of *C. albicans* biology or virulence have identified *SPT6* in their screen. A large scale screen of Tn-7 transposon insertion mutants for those that had an alteration in filamentation phenotype identified 146 genes that affect the yeast to hypha transition, based on altered colony morphology relative to wild-type when grown on YEPD containing 1% serum and/or spider medium (Uhl, et al., 2003). Four individual clones with transposon insertions in *SPT6* were identified in the screen. Three were either hyperfilamentous or had a textured colony morphology on serum agar, while the fourth was less filamentous than wild-type on spider medium. The authors noted that among the 60% of identified genes that had homologues in *S. cerevisiae*, the largest group were involved in transcription and its regulation. Genome wide transcript profiling of *C. albicans* in both a reconstituted human epithelium (RHE) model as well as in smear samples collected from 11 HIV-positive patients with oral thrush identified upregulation of *SPT6* transcription, which persisted at all time points taken over 24 hours in the RHE model (Zakikhany, et al., 2007). This finding supports a role for this gene in the setting of infection, perhaps related to its role in filamentation. Finally, *SPT6* was identified as one of 66 *C. albicans* genes that are preferentially expressed during oral candidiasis in humans using an antibody-based screening approach (Nguyen, et al., 2004). In this approach, pooled sera from 24 HIV-positive patients with active oral thrush were extensively adsorbed against whole cells and varied extracts of a *C. albicans* clinical isolate grown *in vitro*. The adsorbed sera were then used to screen a genomic DNA expression library for reactive clones. The approach was validated in a subset of these genes by showing preferential expression in an HIV-positive patient relative to growth *in vitro*, and localization of gene products to *C. albicans* cells within pseudomembranes using polyclonal antibodies raised against purified antigens (Cheng, et al., 2003). Again, among 58 genes with known or putative function, the largest group (23 genes) was comprised of genes involved in transcription or regulation. In the present study, we have demonstrated that lack of *SPT6* expression resulted in disordered hyphal growth; a finding consistent with a role for this gene product in virulence.

If Spt6p truly has a role analogous to that of *S. cerevisiae* as suggested by the complementation data, its exposure on the hyphal surface and reactivity with scFv5 is difficult to reconcile. One

possibility is that the actual cognate antigen happens to share an epitope with Spt6p that was recapitulated when the cDNA was expressed. Certainly expression of the library in *E. coli* introduces variability in epitope expression relative to the *in vivo* situation. However, the possibility of exposure of Spt6p on the cell surface cannot be excluded, as other cytoplasmic proteins are well documented to be surface exposed for at least part of the cell's life cycle. The glycolytic enzymes, enolase, 3-phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have been shown to be present in the cell wall as well as the cytoplasm, despite the absence of classical secretion signal sequences. Although enolase is not exposed at the cell surface, surface exposure of PGK and GAPDH has been demonstrated (Alloush, et al., 1997; Angiolella, et al., 1996; Gil-Navarro, et al., 1997; Sundstrom and Aliaga, 1994). A more recent study using a proteomic approach with biotinylation to identify surface exposed antigens also identified PGK and GAPDH (Urban, et al., 2003). Another protein, thiol-specific antioxidant-like protein 1 (Tsa1p), was localized to the cell surface in hyphae whereas only nuclear localization was detected in yeast forms. Thus, the localization of this protein varies with growth condition of the cell. Therefore, although not definitive, a dual localization of Spt6p cannot be excluded and will be the focus of ongoing study.

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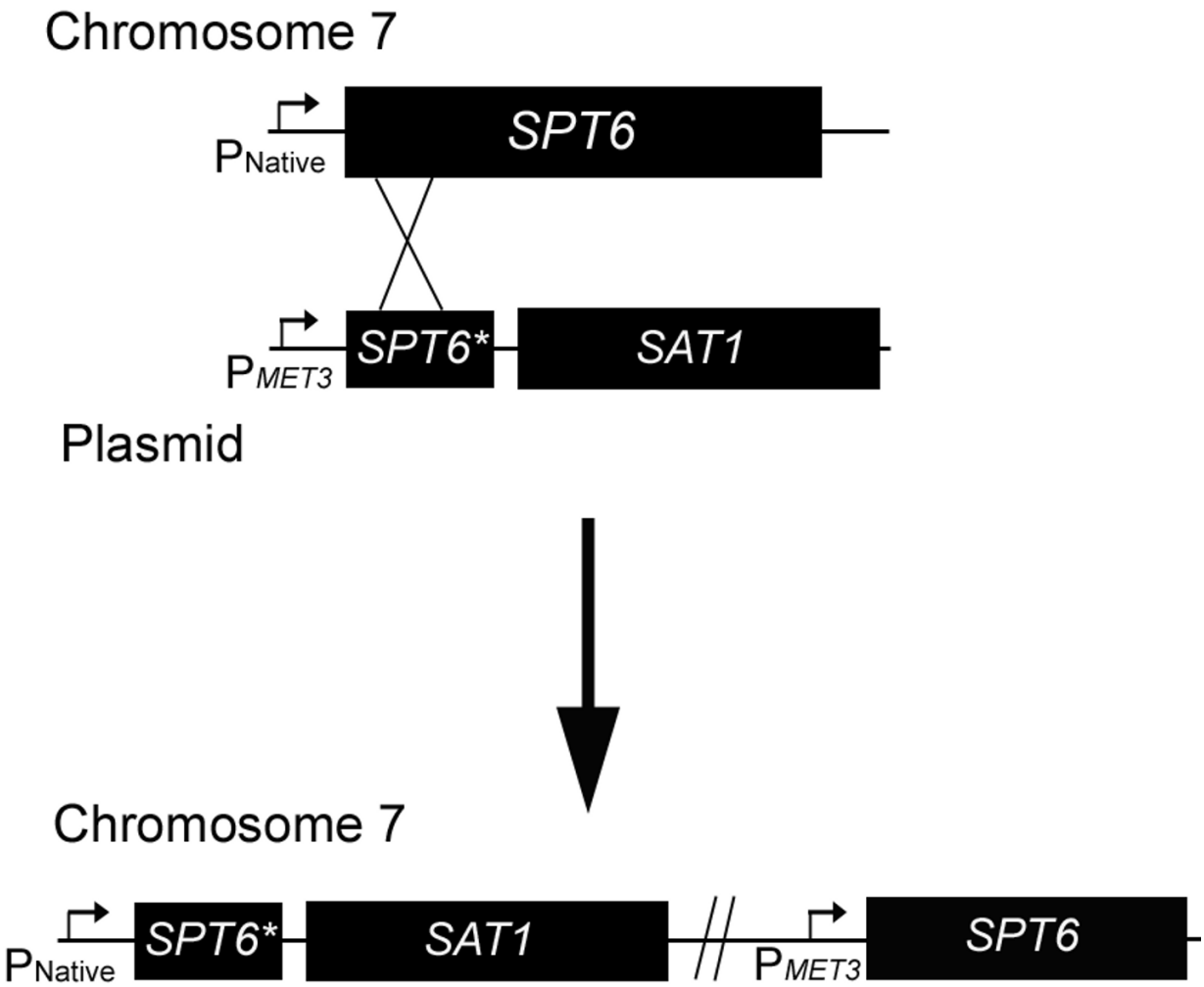

Fig.1. Strategy for construction of *SPT6* reintegrant strain

Because transformation of the Δ*spt6*/Δ*spt6* mutant was inefficient, the heterozygous strain, JMB220, was transformed with the construct depicted. Homologous recombination (depicted by "X") resulted in a truncated *SPT6* gene (*SPT6**) adjacent to the native promoter (P_{Native}), and a full length gene adjacent to the *MET3* promoter (P_{MET3}). The *SAT1* gene was included in the construct to allow for positive selection of transformants. See Materials and Methods for details.

Fig. 2. Comparison of Spt6p SH2 domain among species

The alignment of the amino acid sequences of the Spt6p SH2 domains of *C. albicans* (C.a.), *C. glabrata* (C.g.), and *S. cerevisiae* (S.c.) is depicted. The SH2 domain represents residues 1203 - 1297, or 95 amino acids of the predicted 1402 in the entire protein (7%). Secondary structure elements are indicated above the alignment (cylinders for α helices, arrows for β strands) (Dengl, et al., 2009). Invariant residues are in red and conserved residues are in blue.

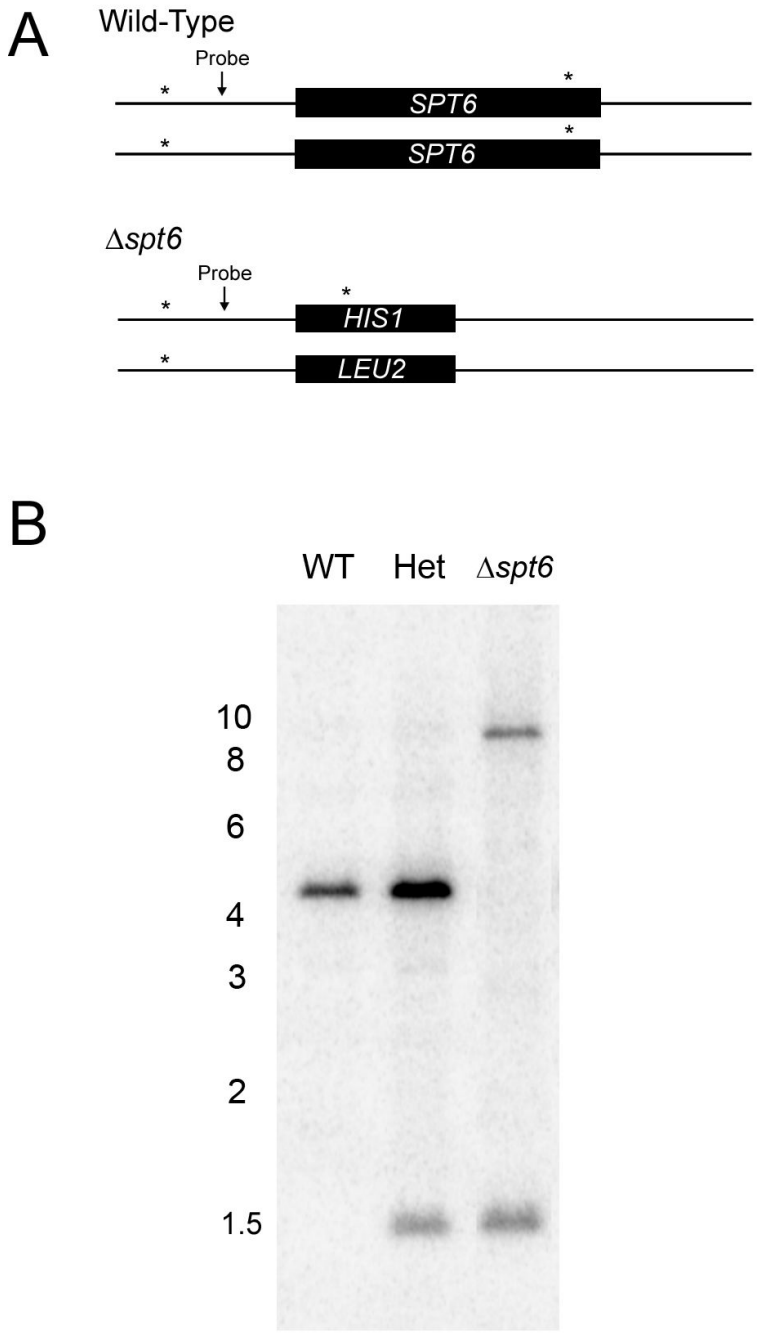

Fig. 3. Southern analysis to confirm homozygous deletion of *SPT6* in JMB31

Genomic DNA was prepared from the wild-type (WT) parent, from the heterozygous strain resulting from replacement of *SPT6* with *HIS1* (JMB220, Het), and from the strain with both copies of *SPT6* replaced by *HIS1* and *LEU2* respectively (JMB31, Δ*spt6*). Genomic DNA was digested with *Sca*I and analyzed by Southern blot. Recognition sites for this restriction enzyme exist within the *SPT6* ORF, as well as the neighboring upstream and downstream sequences. The gene deletion strategy was predicted to eliminate the *Sca*I site both within and downstream to the ORF. Panel A shows the predicted *Sca*I recognition sites (*) and the binding location of the DNA probe. Digestion of wild-type DNA was predicted to yield a 4.2 kb fragment and digestion following replacement with *HIS1* predicted a 1.5 kb fragment. *SPT6* is adjacent to

the telomere of chromosome 7. Because the *LEU2* marker does not contain a *Sca*I site, and the only *Sca*I site in the region 3′ to the gene was deleted with *SPT6*, the fragment containing the *LEU2* marker was predicted to extend to the telomeric region of the chromosome; ~ 10 kb. Panel B demonstrates the predicted banding pattern, confirming homozygous deletion of *SPT6*. Positions of the relevant molecular weight markers (in kb) are depicted.

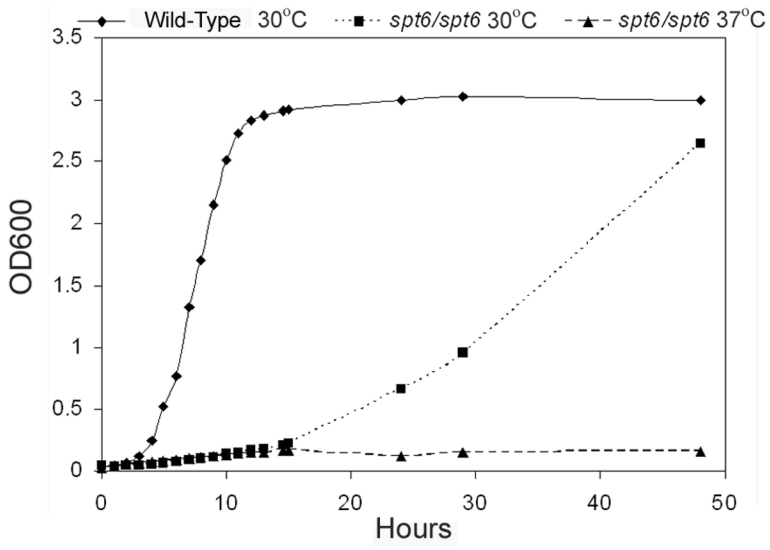

Fig. 4. Growth of Δ*spt6*/Δ*spt6* mutant

The Δ*spt6*/Δ*spt6* mutant and its wild-type parent were grown in YPD media at 30°C and 37°C and growth was measured over time by optical density at 600 nm (OD600). The mutant demonstrated slower growth than the wild-type at 30°C and no growth at 37°C.

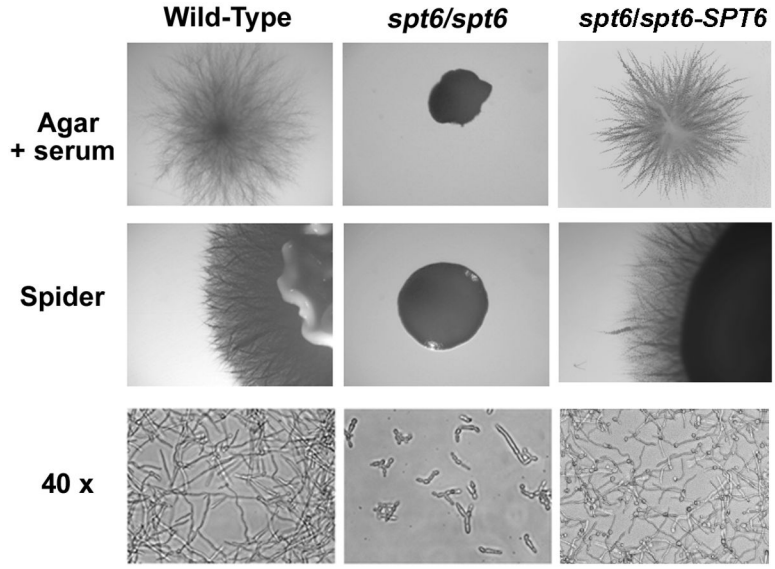

Fig. 5. Hyphal phenotype of Δ*spt6*/Δ*spt6* mutant

Wild-type and mutant cells were grown on the indicated media. In addition to markedly smaller colony size, the mutant generated no detectable hyphae under any conditions. Using phase contrast microscopy (40x), stunted filamentous forms were seen with no true hyphae. The *SPT6* reintegrant strain (Δ*spt6*/*spt6*-*SPT6*) resembled the wild-type phenotype.

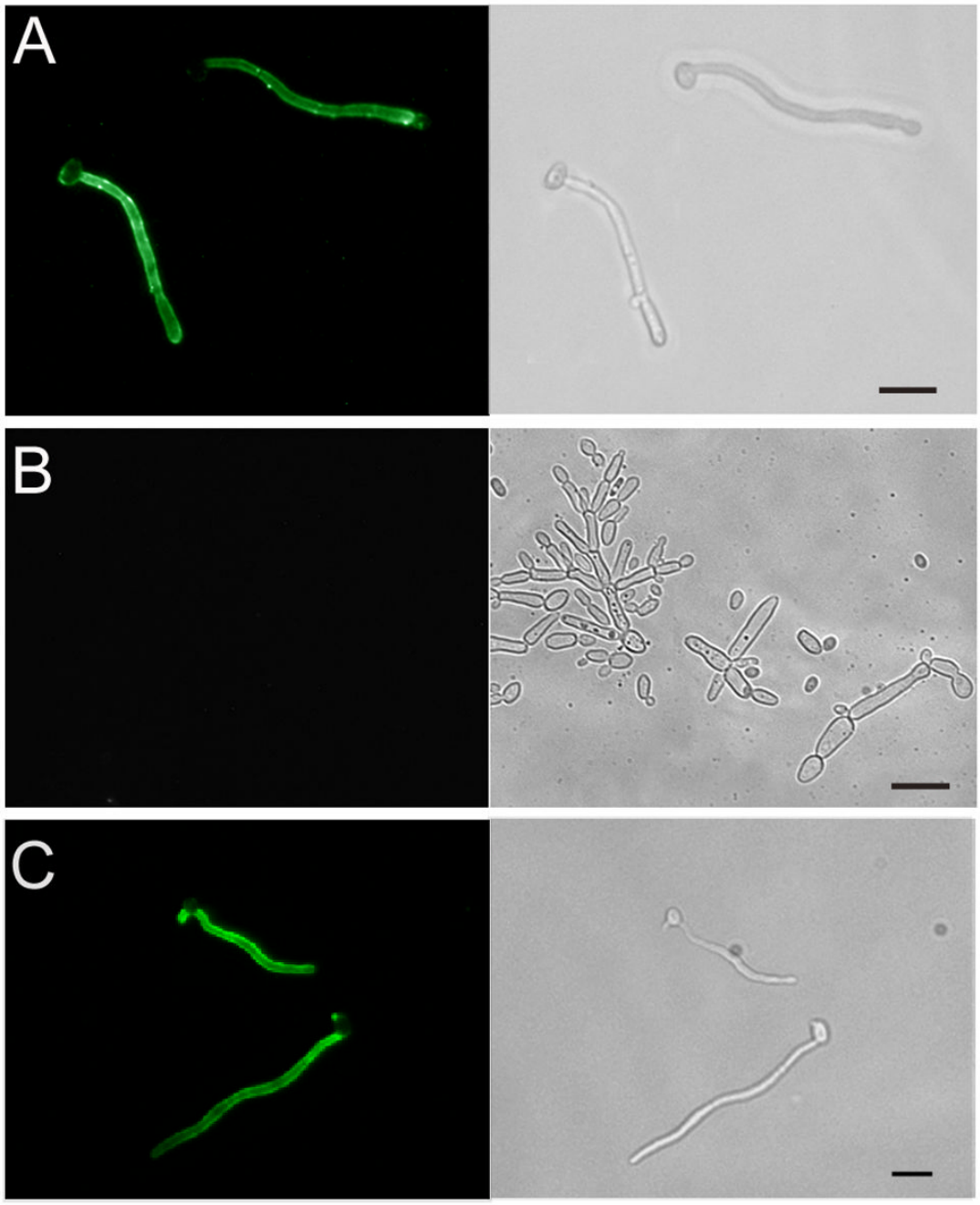

Fig. 6. Indirect immunofluorescence assay (IFA) of wild-type and Δ*spt6*/Δ*spt6* mutant probed with scFv5

Wild-type (Panel A), Δ*spt6*/Δ*spt6* mutant (Panel B), and *SPT6* reintegrant cells (Panel C) were induced to form germ tubes and incubated with scFv5 followed by an appropriate fluorochrome labeled secondary antibody. Fluorescence and phase contrast photomicrographs of the same representative microscopic fields are depicted. The typical hyphae-specific binding of scFv5 to wild-type is seen, whereas no binding is detected to the mutant. The reintegrant strain showed binding with a wild-type pattern. Bar – 10 μm.

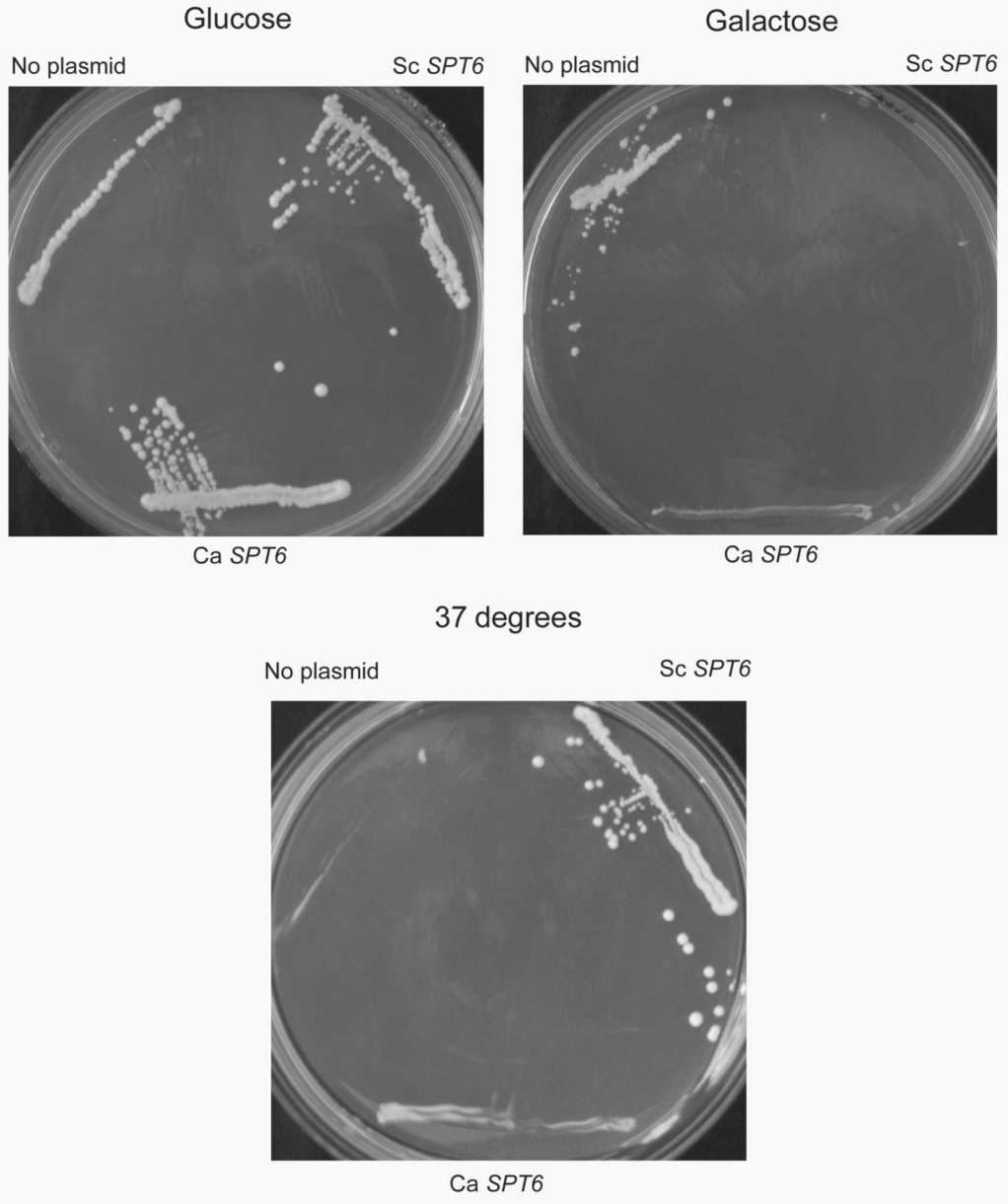

Fig. 7. Complementation of *spt6* in *S. cerevisiae*

FY137 is a *S. cerevisiae* strain containing mutations in *HIS4* and *LYS2* that normally would make the strain auxotrophic for histidine and lysine. However, due to a recessive suppressor mutation in *SPT6* (*spt6-140*), the phenotype of this strain is His+, Lys+. The strain also does not grow at 37°C. In the upper portion of the figure, growth of FY137 containing empty vector, *S. cerevisiae* (*Sc*) *SPT6* or *C. albicans* (*Ca*) *SPT6* under control of a galactose inducible promoter is depicted on His drop out media containing glucose or galactose at 30°C. Because wild-type *SPT6* is not expressed on glucose, the strain grows normally. Induction of *SPT6* expression by galactose leads to return to the His- phenotype, and the *C. albicans* gene has a similar effect to that seen with *S. cerevisiae SPT6*. In the lower portion of the figure, growth of the same strains on galactose at 37°C is shown. The temperature sensitive phenotype of FY137 is partially corrected by expression of *C. albicans SPT6*.

TABLE 1

Strains used in this study

Strain	Genotype	Source
C. albicans		
SC5314	Wild-type clinical isolate	(Gillum, et al., 1984)
SN87	*leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm^{434} IRO1/iro1Δ::imm^{434}*	(Noble and Johnson, 2005)
JMB220	*spt6Δ::C.d HIS1/SPT6 leu2Δ/leu2Δhis1Δ/his1Δ URA3/ura3Δ::imm^{434} IRO1/iro1Δ::imm^{434}*	This study
JMB31	*spt6Δ::C.d HIS1/spt6Δ::C.m.LEU2 leu2Δ/leu2Δ his1Δ/his1Δ URA3/ ura3Δ::imm^{434} IRO1/iro1Δ::imm^{434}*	This study
JMB222	*spt6Δ::C.d HIS1/spt6*-SPT6 leu2Δ/leu2Δ his1Δ/his1Δ URA3/ ura3Δ::imm^{434} IRO1/iro1Δ::imm^{434}*	This study
S. cerevisiae		
FY137	*MATa ura3, his4-912δ, lys2-1288spt6-140*	(Hartzog, et al., 1998)

spt6* represents a truncated gene, containing the first 615 bp of the 4206 bp ORF

TABLE 2

Oligonucleotides used in this study

Name	Sequence[a]
1	GCCCACGATTATCTCAACTTTATCC
2	cacggcgcgcctagcagcggACACCTCTTTCTCTCTTGTTGGGGTT
3	gtcagcggccgcatccctgcTTGTTGTCGTAGTGGAAGGTGATTG
4	TTATGGGACAAACAAGAACTCGAAA
5	GACATACAAATCCTTCCAATGGTCA
6	ATTAGATACGTTGGTGGTTC
7	AGAATTCCCAACTTTGTCTG
8	CCTCATTGAAAATCCAATGGAGCAA
9	AACACAACTGCACAATCTGG
10	AAACTTTGAACCCGGCTGCG
11	CATTTCGAGGTTGTGTGCTG
12	ACATCTTGACCGCCTGCTT
13	AGAGGGTGGTCTCTGGGA
14	GGGGGGGCCCGGGGCAATAAGGTTGAGTGA
15	GGGGCTCGAGACGGCGACAGATGGTTAATG
16	GGGGATCCGCGCAATACACGACCTATATGATG
17	GGCCACTATTTTCCAATAAGAGCTCC
18	GGAGCTCTTATTGGAAAATAGTGGCC
19	GGCTGCAGCGACGTATCAATTCGGGGACCTTGC
20	CAATCAAAGGTGGTCCTGCAG
21	GTAAATACCCTCCCCGGATCC
22	CAAACTGAACAACTTGTCCC
23	GGGACTAGTATGATGAAAAAAAAAATCTCTGCC
24	GGGCTCGAGCTAATAATACCCCGTGTTATATGACT

[a] Lowercase letters indicate exogenous sequences used for fusion PCR reactions as described previously (Noble and Johnson, 2005). Underlined sequences are restriction sites described in the text.